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Original article

Novel non-covalent thrombin inhibitors incorporating P_1 4,5,6,7-tetrahydrobenzothiazole arginine side chain mimetics

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Dedicated to Professor Branko Stanovnik on occasion of his 65th birthday

Abstract

The design, synthesis and biological activity of a series of novel non-covalent D-Phe-Pro-Arg motif-based thrombin inhibitors incorporating 4,5,6,7-tetrahydrobenzothiazol-2-amine as a novel arginine surrogate are described. Compound **9**, the most potent in the series of thrombin inhibitors, exhibited an in vitro K_i of 128 nM and 342-fold selectivity against trypsin. The binding mode of this novel class of thrombin inhibitors in the enzyme active site, based on the X-ray crystal structure of compound **9** co-crystallized with human α-thrombin, is discussed.

Keywords: Thrombin inhibitors; Peptidomimetics; Arginine side chain mimetic; 4,5,6,7-Tetrahydrobenzothiazole

1. Introduction

Thrombin, a trypsin-like serine protease, is a key enzyme in the blood coagulation cascade [1]. Serving as the terminal enzyme of the blood coagulation pathway, it catalyzes the conversion of fibrinogen to fibrin and the cross-linking of fibrin to form the blood clot. Thrombin is also a potent stimulant of platelet aggregation. Thus, it plays a major role in development of thromboembolic diseases, which remain the leading cause of morbidity and mortality in developed countries. Inhibition of thrombin is therefore an attractive therapeutic target for intervention in thrombosis [2]. During the last decade, the search for orally bioavailable, potent and selective low molecular weight, active site-directed thrombin inhibitors has emerged as one of the most intensive areas of investigation in drug discovery [3].

Numerous thrombin inhibitors, such as inogatran [4] and melagatran [5], have been developed based on the D-Phe-Pro-Arg sequence [6], which mimics thrombin's natural substrate, fibrinogen. A general strategy in the design of these tripeptidomimetic thrombin inhibitors [3b] continues to be the replacement of individual amino acids in the D-Phe-Pro-Arg motif by their mimetic compounds, in order to achieve an optimal combination of potency, selectivity versus similar serine proteases and oral bioavailability. Among this class of thrombin inhibitors, most research has been devoted to compounds lacking the C-terminus of the arginine residue, which were obtained by incorporating various arginine side chain mimetic groups [7]. In order to overcome the high basicity of the P_1 guanidine, amidine and aliphatic amine moieties which impair oral bioavailability of thrombin inhibitors, various amino heterocycles were investigated as the P_1 arginine side chain mimetics [3]. Among these, some bicyclic aromatic (amino)heterocycles, e.g., 1-isoquinolinamine [8], 3-benzisoxazolamine [9], indole [10], benzimidazole [10], indazole [10,11], imidazo[1,2-*a*]pyridine [12] and pyrrolo[3,2-*b*]pyridine [13] were recently employed as P_1 moieties in tripeptidomimetic thrombin inhibitors.

Abbreviations: Chx, β-cyclohexylalanine; BTZ, 4,5,6,7-tetrahydrobenzothiazole.

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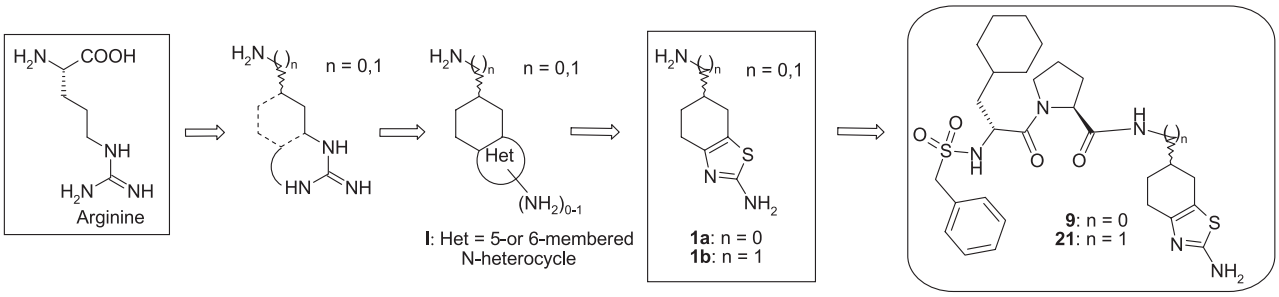


Fig. 1. Evolution of 4,5,6,7-tetrahydrobenzothiazol-2-amine arginine side chain mimetics and novel thrombin inhibitors which incorporate them.

Our approach to the design of thrombin inhibitors based on the D-Phe-Pro-Arg sequence was focused mainly on replacing the arginine moiety with less basic, conformationally restricted, partially saturated heterobicyclic arginine side chain mimetics **I** (Fig. 1). These contain a five- or six-membered *N*-heterocyclic ring optionally substituted by an amino group mimicking the guanidino moiety of arginine, annulated to a cyclohexane ring mimicking the arginine trimethylene side chain. We speculated that the bulky and lipophilic cyclohexane ring could confer selectivity for thrombin against trypsin, since the S_1 pocket in thrombin is slightly larger and more hydrophobic than the one in trypsin. Moreover, a conformationally flexible cyclohexane ring in the P_1 part of the inhibitor could allow a better fit of the inhibitor in the enzyme active site than inhibitors incorporating rigid and planar P_1 aromatic heterobicycles.

In this paper, we report the design, synthesis, in vitro biological activity, and a binding mode of thrombin inhibitors incorporating 4,5,6,7-tetrahydrobenzothiazol-2-amine as a P_1 moiety.

2. Chemistry

Synthetic routes to 4,5,6,7-tetrahydrobenzothiazol-2-amine arginine side chain mimetics **1a** and **1b** for use as P_1 moieties are outlined in Figs. 2 and 3, respectively. 4,5,6,7-Tetrahydrobenzothiazole-2,6-diamine (**1a**) was prepared from *N*-(4-oxocyclohexyl)-acetamide (**2**) [14] by bromination, subsequent Hantzsch thiazole synthesis using thiourea as an S–C–N synthon [15] and final acid hydrolysis of the acetamido group of **4**. Similarly, bromination of *N*-[(4-oxocyclohexyl)methyl]-acetamide (**5**) [16] and reaction of the resulting bromoketone **6** with thiourea afforded the annulated thiazole **7** which, on hydrolysis, gave 6-aminomethyl-4,5,6,7-tetrahydrobenzothiazol-2-amine (**1b**).

Compounds **9–18** and **21** were prepared by coupling arginine surrogates **1a, 1b** and benzothiazole-2,6-diamine to the optionally protected P_2-P_3 moiety **8**, using *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT) as amide bond forming reagents. To obtain inhibitors with a free N-terminus, the corresponding *N-tert*-butyloxycarbonyl protected compounds were cleaved by hydrogen chloride in glacial acetic acid solution (Fig. 4). Compounds **19** and **20** were prepared similarly by coupling of *N*-benzylsulfonyl-D-phenylalanine with P_2-P_1 moieties **23** and **24**. The target inhibitors were purified with column

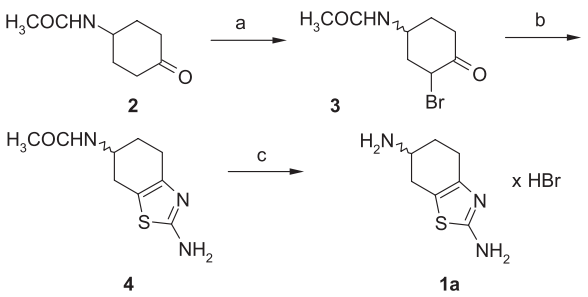


Fig. 2. Reagents and conditions: (a) Br_2, AcOH, 60 °C, 1 h; (b) thiourea, AcOH, reflux, 1.5 h; (c) 47% aq. HBr, reflux, 24 h.

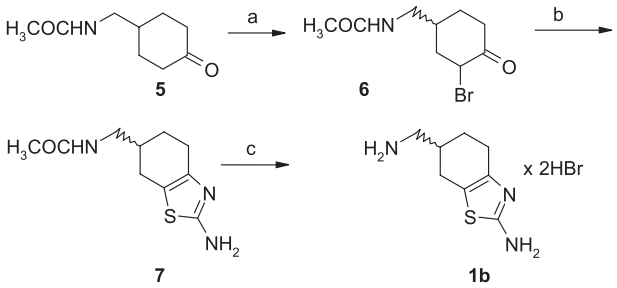


Fig. 3. Reagents and conditions: (a) Br_2, CH_2Cl_2, rt, 1 h, then reflux, 1 h; (b) thiourea, EtOH, reflux, 2 h; (c) 47% aq. HBr, reflux, 16 h.

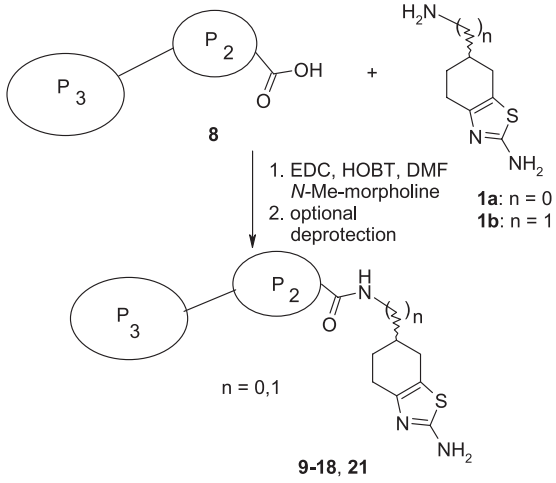


Fig. 4. General scheme for synthesis of compounds **9–18** and **21**.

chromatography and obtained as diastereomeric mixtures with respect to C-6 of the 4,5,6,7-tetrahydrobenzothiazole ring.

3. Pharmacology

The ability of the new thrombin inhibitors to inhibit the enzymatic activity of thrombin, trypsin and factor Xa was measured by amidolytic assay and expressed as inhibitory constants (K_i values), determined according to Cheng and Prusoff based on IC_{50} [17a], or from a relation between reaction velocity in the absence and presence of inhibitor using the relevant K_m [17b]. The selectivity for thrombin over trypsin was expressed as the ratio $K_{i(trypsin)}/K_{i(thrombin)}$. Inhibitors were also tested on normal pool plasma with standard clotting assays, including thrombin time (TT), activated partial thromboplastin time (APTT) and prothrombin time (PT) assays, which were used as qualitative in vitro indicators of potential antithrombotic activity.

4. Results and discussion

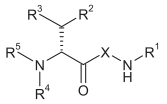
The results are summarized in Table 1. Several of these compounds showed good in vitro potency against thrombin, with desirable selectivity towards trypsin. Inhibitor **9**, possessing 4,5,6,7-tetrahydrobenzothiazol-2,6-diamine to fill the selectivity pocket, proline in the central part and a benzylsulfonyl group attached to D-cyclohexylalanine as the P_3 part of the molecule, was the most potent with a K_i value of 128 nM and a 342-fold selectivity for thrombin over trypsin. It doubled TT, APTT and PT at concentrations of 16, 59 and 114 μM, respectively. Contrary to our expectation, a methylene linker between the cyclohexane ring of a heterocycle and the amino group, which might allow a substantial rotational freedom of the P_1 part, was not beneficial for inhibitory activity against thrombin. Thus, compound **21** exhibited threefold less inhibitory potency and threefold lower selectivity for thrombin over trypsin than inhibitor **9**. This is probably due to the increased length of the molecule, which is responsible for a shift of the P_2 and P_3 groups from the sites of optimal interaction with the active site residues. Compounds **9** and **21** were almost equipotent in all clotting assays. Interestingly, the potency of compound **9**, with D-cyclohexylalanine in the P_3 part, exceeded the potency of **12**, with D-phenylalanine, in all the assays by a factor of 5–6. Based on the calculated log *P* values (4.20 ± 0.86 for compound **9** and 3.57 ± 0.89 for compound **12**) [18], it is anticipated that the potency of **9** is affected to a greater extent than that of **12** by non-specific binding to plasma proteins.

The replacement of D-phenylalanine in compound **12** by D-3,4-dichlorophenylalanine resulted in a twofold improvement of inhibition constant (**10**; K_i = 352 nM). The potency of compound **16**, lacking a P_3 benzylsulfonyl group, was 10-fold lower (K_i = 3.392 μM) than that of **10**, indicating an important contribution of the benzylsulfonyl group to the inhibitory activity of compound **10**. Only slightly higher inhibitory potencies against human thrombin were observed in **14** (K_i = 1.100 μM) and **15** (K_i = 2.360 μM) featuring a D-diphenyl moiety at the P_3 part of the molecule.

Compounds **11** and **13**, which can be regarded as *N*-carboxymethyl analogs of **9**, displayed submicromolar inhibition constants for thrombin (**11**: K_i = 560 nM; **13**: K_i = 800 nM), proving that the *N*-benzylsulfonyl group of **9** undergoes a more favorable interaction with the enzyme active site than the *N-tert*-butyloxycarbonyl-methyl and *N*-carboxymethyl groups of **11** and **13**, respectively. Inhibitor **11** displayed the highest, 810-fold, selectivity for thrombin over trypsin of all the compounds studied.

In an attempt to evaluate the influence of planarity of the heterobicyclic P_1 moiety, benzothiazole-2,6-diamine was ex-

Table 1
Inhibitory activities of compounds **9-21**: P_1, P_2 and P_3 modifications



Compd	R^5	R^4	R^3	R^2	X	R^1	K_i (μM) Thrombin	Trypsin	FXa	Selectivity Thrombin/Trypsin
9	H	SO_2CH_2Ph	H	Chx	L-Pro		0.128	43.8	> 75.4	342
10	H	SO_2CH_2Ph	H	3,4-Cl_2-Ph	L-Pro		0.352	> 68.3	> 75.4	>194
11	Boc	CH_2COO-*t*Bu	H	Chx	L-Pro		0.560	453.5	88.4	810
12	H	SO_2CH_2Ph	H	Ph	L-Pro		0.621	> 68.3	> 75.4	>110
13	H	CH_2COOH	H	Chx	L-Pro		0.800	19.5	84.4	24
14	H	Boc	Ph	Ph	L-Pro		1.100	*a*	69.9	ND
15	H	H	Ph	Ph	L-Pro		2.360	153	82.9	65
16	H	H	H	3,4-Cl_2-Ph	L-Pro		3.392	> 68.3	> 75.4	> 20
17	H	SO_2CH_2Ph	H	Ph	Gly		> 35.1	> 68.3	> 75.4	ND
18	H	SO_2CH_2Ph	H	Ph	L-Ala		> 35.1	> 68.3	> 75.4	ND
19	H	SO_2CH_2Ph	H	Ph	L-Pro		> 35.1	> 68.3	> 75.4	*a*
20	H	SO_2CH_2Ph	H	Ph	Gly		5.100	> 68.3	> 75.4	> 13
21	H	SO_2CH_2Ph	H	Chx	L-Pro		0.370	39.9	65.4	108

[a] Due to precipitation measurements were not possible.

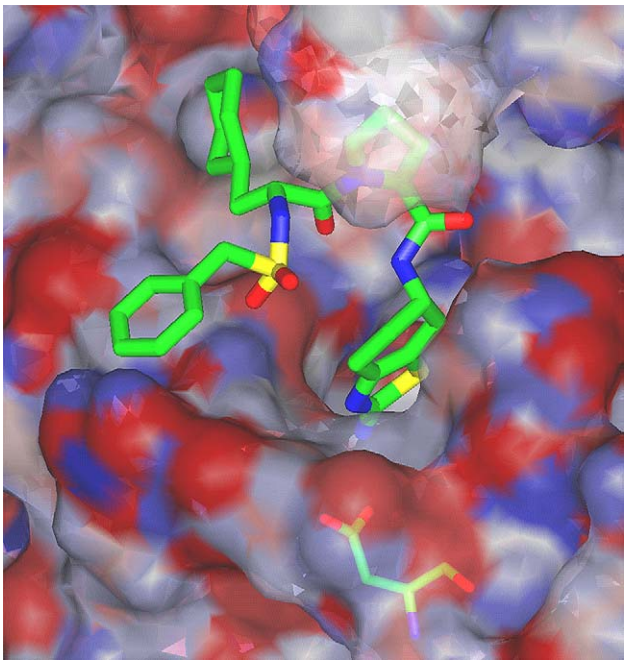


Fig. 5. Connolly surface map of the X-ray structure of the human α-thrombin—inhibitor **9** complex at 1.9 Å resolution. Inhibitor **9** is shown as sticks. Colors green, blue, red and yellow identify carbon, nitrogen, oxygen and sulfur, respectively. Contact with Asp 189 is shown.

plored as an arginine surrogate instead of 4,5,6,7-tetrahydrobenzothiazol-2-amine. The resulting compound **19** was devoid of inhibitory activity against thrombin (K_i > 35.1 μM) and trypsin (K_i > 68.3 μM). A great drop in potency of **19** (calcd. pK_a of N3 = 5.13 ± 0.30) [18] with regard to the corresponding tetrahydro derivative 12 (calcd. pK_a of N3 = 6.14 ± 0.40) [18] cannot be interpreted only by difference in basicity of P_1 moiety of both compounds but also in terms of higher flexibility of compound **12**. Thus, the activity of 19 was improved by replacing proline by glycine in the P_2 part of the inhibitor to give **20** with an inhibition constant K_i = 5.10 μM for thrombin. The beneficial effect of replacing proline in **19** by glycine can be explained by diminished rigidity of inhibitor **20** in the P_2 part, allowing a better overall fit of the molecule in the thrombin active site. In contrast, substitution of proline by glycine or alanine in a P_1 4,5,6,7-tetrahydrobenzothiazol-2-amine containing inhibitor **12**, resulting in compounds **17** or **18**, was detrimental to anti-thrombin activity (K_i > 35.1 μM).

All the thrombin inhibitors listed in Table 1 were also selective with respect to inhibition of factor Xa. Inhibition of the latter appears to be in the same range for all the compounds tested, and is hardly affected by any of the replacements studied.

The potency and selectivity of inhibitor **9** prompted us to determine its precise binding conformation in the thrombin active site by X-ray crystallography. Fig. 5 shows the overall binding mode of **9** in the thrombin active site with the partially saturated heterobicyclic arginine side chain mimetic occupying the S_1 selectivity pocket, the proline ring nestling under the YPPW loop, and the cyclohexane ring occupying the aryl binding site. Altogether, inhibitor **9** forms six hydrogen bonds to the surrounding residues. As shown in Fig. 6, the 2-amino-4,5,6,7-tetrahydrobenzothiazole NH_2 group forms a hydrogen bond with Oδ1 Asp189 (2.71 Å) and O Ala190 (3.35 Å), and is additionally hydrogen bonded through a water mediated hydrogen bond to the backbone carbonyl oxygens of Phe227 and Trp215. The crystals of thrombin with the inhibitor **9** bound in the active site were prepared using a diastereomeric mixture of **9**, with respect to the stereogenic center at C6 of the 4,5,6,7-tetrahydrobenzothiazole ring, whereas the model of **9** that fits best into the final 2Fo-Fc density has the *S* configuration at C6 and equatorial orientation of the chain attached to C6. Consequently, the sulfur atom of the benzothiazole ring is directed towards Val213. The S2 site is occupied by proline, with its carbonyl group forming a hydrogen bond to both the Oγ Ser195 (2.80 Å) and Nε2 His57 (3.06 Å) of the catalytic triad and to Gly193 via a water molecule. The P3 cyclohexyl

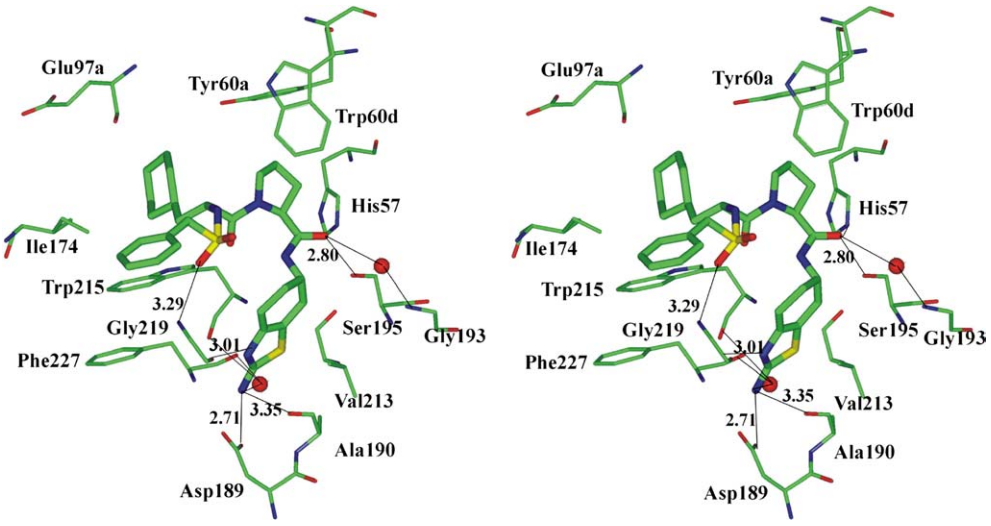


Fig. 6. Schematic representation of inhibitor **9** bound in the active site of thrombin. Thin lines indicate hydrogen bonds. Water molecules are represented as red spheres. Distances are given in Ångstroms.

ring lies in the lipophilic aryl binding site. The electron density of the benzyl group was contoured at 0.8σ. It points loosely towards the solvent, which implies that it is not stably bound. Thus, the contribution to affinity of the ligand is not favorable. Crystallographic analysis of **9** bound in the thrombin active site indicated that one of the N-terminal sulfonamide oxygens participates in a hydrogen-bonding interaction with the NH of Gly219 (3.29 Å) [19].

5. Conclusion

In summary, we have prepared a series of thrombin inhibitors incorporating P_1 4,5,6,7-tetrahydrobenzothiazol-2-amine moiety as a novel arginine side chain mimetic. Variations in the P_2 and P_3 parts led to thrombin inhibitor **9** which exhibited an in vitro K_i of 128 nM and 342-fold selectivity against trypsin. The results of the crystal structure of **9** complexed in the thrombin active site will be used for further structure-based design of improved D-Phe-Pro-Arg sequence based thrombin inhibitors. The studies concerning oral bioavailability of these thrombin inhibitors will be reported in due course.

6. Experimental protocols

6.1. Chemistry

6.1.1. Materials and methods

Chemicals were purchased from Aldrich Chemical Co., Fluka and Synthetech and used without further purification. THF was kept over sodium and distilled immediately prior to use. Analytical TLC was performed on Merck silica gel (60 F 254) plates (0.25 mm). Visualization was effected with ultraviolet light, ninhydrin and 2,4-dinitrophenylhydrazine. Column chromatography was carried out on Florisil® (particle size 100–200 mesh) and silica gel 60 (particle size 240–400 mesh). Melting points were determined on a Reichert hot stage microscope and are uncorrected. ^{1}H-NMR spectra were recorded on a Bruker AVANCE DPX$_{300}$ spectrometer in $CDCl_3$ or DMSO solution with TMS as the internal standard. In proline containing compounds **9–16** and **21**, due to *cis/trans*-isomerism of the proline peptide bond and due to the presence of stereogenic center at C-6 the NMR spectra are very complex and therefore, only ^{1}H-NMR spectrum of **9** is reported [20, 21a]. IR spectra were obtained on a Perkin–Elmer 1600 FT-IR spectrometer. Microanalyses were performed on a Perkin–Elmer C, H, N analyzer 240 C. Analyses indicated by the symbols of the elements or functions were within +0.4% of the theoretical values. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. Chemical names were generated using ACD/Name software.

6.1.2. N-*(Benzylsulfonyl)-D-phenylalanyl-L-alanine; general procedure for the synthesis of P_3–P_2 fragments* ***8***

6.1.2.1. N-*Benzylsulfonyl-D-phenylalanine.* A solution of benzylsulfonyl chloride (5.91 g, 31 mmol) in dioxane (100 ml) was added dropwise to a stirred mixture of D-phenylalanine hydrochloride (5.44 g, 27 mmol) and NaOH (1 M, 27 ml) in dioxane (100 ml) at 0 °C. After the addition was complete, the mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure, the residue was dissolved in water (50 ml), acidified to pH 2 with saturated aqueous $KHSO_4$ and extracted with ethyl acetate (3 × 50 ml). The organic phase was washed with brine, dried over Na_2SO_4 and evaporated in vacuum to give a yellow oil; yield: 3.88 g (45%); IR (NaCl-Film): *ν* 3262, 3064, 2931, 1731, 1496, 1456, 1374, 1322, 1264, 1153, 1127, 1043, 956, 846, 782, 698 cm$^{-1;}$ ^{1}H-NMR (300 MHz, DMSO-d_6): 2.78–3.06 (ABX-system, 2H, J_{AB} = 13.66 Hz, J_{AX} = 8.85 Hz, J_{BX} = 5.65 Hz, CH_2Ph), 3.92–4.09 (m, 3H, $PhCH_2SO_2$ + CH), 7.17–7.37 (2 × m, 10H, 2 × Ph), 7.65 (d, 1H, *J* = 8.67 Hz, –SO_2NHCH–), 12.5 (s br, 1H, COOH) ppm.

6.1.2.2. N-*Benzylsulfonyl-D-phenylalanyl-L-alanine methyl ester.* To a solution of *N*-benzylsulfonyl-D-phenylalanine (1.59 g, 5 mmol) and L-alanine methyl ester hydrochloride (0.63 g, 4.5 mmol) in *N,N*-dimethylformamide (12.5 ml), 1-hydroxybenzotriazole (0.68 g, 5 mmol) and, after adjusting the pH of the resulting solution to 8 with *N*-methylmorpholine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.96 g, 5 mmol) were added. The reaction mixture was stirred overnight at room temperature, the solvent was evaporated in vacuo and the residue partitioned between EtOAc (50 ml) and saturated aqueous $NaHCO_3$ (3 × 50 ml). The organic layer was washed with brine (2 × 50 ml), dried over $MgSO_4$, filtered and the solvent was removed in vacuo to give 1.73 g (95%) of a pale yellow foam; IR (KBr): *ν* 3281, 2952, 1736, 1648, 1543, 1456, 1317, 1221, 1151, 1125, 952, 790, 749, 698, 548 cm^{-1}; MS (FAB): 405 (MH^+, 47%), 91 (100%); ^{1}H-NMR (300 MHz, DMSO-d_6): 1.19 (d, 3H, *J* = 7.16 Hz, $CHCH_3$), 2.86 (ABX-system, 2H, J_{AB} = 13.56 Hz, J_{AX} = 9.04 Hz, J_{BX} = 6.03 Hz, CH_2Ph), 3.58 (s, 3H, $COOCH_3$), 4.01 (AB system, 2H, J_{AB} = 13.56 Hz, $PhCH_2SO_2$), 4.15–4.36 (m, 2H, CH–Ala, CH–Phe), 7.19–7.35 (m, 10H, 2 × Ph), 7.67 (d, 1H, *J* = 9.04 Hz, –SO_2NHCH–), 8.61 (d, 1H, *J* = 7.16 Hz, CONH) ppm.

6.1.2.3. N-*Benzylsulfonyl-D-phenylalanyl-L-alanine.* To a solution of *N*-benzylsulfonyl-D-phenylalanyl-alanine methyl ester (1.60 g, 3.96 mmol) in methanol/water (1:1, 100 ml), lithium hydroxide (2.2 M, 2.38 ml) was added dropwise during 0.5 h to keep pH between 12 and 13. The mixture was stirred overnight, then pH was adjusted to 7 with $KHSO_4$ and methanol was evaporated under reduced pressure. The residual aqueous solution was acidified to pH 2 with $KHSO_4$ and extracted with ethylacetate (3 × 50 ml).

The combined organic extracts were washed with brine (2 × 75 ml), dried Na_2SO_4, filtered and evaporated in vacuum to give 896 mg (58%) of a pale yellow foam; IR (KBr): *v* 3321, 2930, 1734, 1654, 1541, 1497, 1456, 1321, 1152, 1031, 950, 783, 747, 699, 621 cm^{-1}; MS (FAB): 391 (MH^+, 100%); ^{1}H-NMR (300 MHz, DMSO-d_6): 1.15–1.21 (m, 3H, Ala–CH_3), 2.86 (ABX-system, 2H, J_{AB} = 13.56 Hz, J_{AX} = 9.04 Hz, J_{BX} = 6.03 Hz, CH_2Ph), 4.03 (AB system, 2H, J_{AB} = 13.56 Hz, PhCH_2SO_2), 4.17–4.35 (m, 2H, CH–Ala, CH–Phe), 7.20–7.33 (m, 10H, 2 × Ph), 7.65 (d, 1H, *J* = 9.04 Hz, –SO_2NHCH–), 8.50 (d, 1H, *J* = 7.53 Hz, CONH), 12.62 (s br, 1H, COOH) ppm; Anal. $C_{19}H_{22}N_2O_5S$ (C, H, N).

Using the same general procedure, *N*-(benzylsulfonyl)-D-(β-cyclohexyl)alanyl-L-proline, *N*-(benzylsulfonyl)-D-phenylalanyl-glycine, *N*-(benzylsulfonyl)-D-phenylalanyl-L-proline, and *N*-(benzylsulfonyl)-D-(3,4-dichloro)phenyl-alanyl-L-proline were prepared.

*6.1.3. (2S)-N-(2-Amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)-1-{(2R)-2-[(benzylsulfonyl)-amino]-3-cyclohexyl-propanoyl}-2-pyrrolidinecarboxamide (**9**); general procedure for the synthesis of compounds **9–12, 14, 17, 18** and **21–22** by coupling of P_3–P_2 fragments **8** with 4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine (**1a**), 6-(aminomethyl)-4,5,6,7-tetrahydro-1,3-benzothiazol-2-amine (**1b**) and 1,3-benzothiazole-2,6-diamine*

To a solution of *N*-(benzylsulfonyl)-D-(β-cyclohexyl)-alanyl-L-proline (250 mg, 0.59 mmol) and 4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrobromide (178 mg, 0.54 mmol) in *N,N*-dimethylformamide (1.5 ml), 1-hydroxybenzotriazole (80 mg, 0.59 mmol) and, after adjusting the pH of the resulting solution to 8 with *N*-methylmorpholine, 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (113 mg, 0.59 mmol) were added. The reaction mixture was stirred overnight at room temperature, the solvent was evaporated in vacuo and the residue partitioned between EtOAc (10 ml) and saturated aqueous $NaHCO_3$ (3 × 10 ml). The organic layer was washed with brine (2 × 10 ml), dried over $MgSO_4$, filtered and the solvent was removed in vacuo. The crude product was purified by column chromatography (silica gel, $CHCl_3$/MeOH = 9:1) to give **9** as a faint brown crystalline solid; yield: 167 mg (54%); mp 127–130 °C. IR (KBr): *v* 3357, 2924, 2848, 1638, 1523, 1448, 1311, 1125, 754, 697, 546 cm^{-1}. MS (FAB): 574 (MH^+, 100%). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 0.70–0.98 (m, 2H, Chx), 1.00–1.28 (m, 3H, Chx), 1.30–1.52 (m, 2H, Chx), 1.53–2.10 (m, 9H, 3 × CH_2–Chx, βCH–Pro, γ-CH_2–Pro), 2.20–2.60 (m, 6H, 3 × CH_2–BTZ), 2.60–2.74 (m, 1H, CH), 3.38–3.48 (m, 1H, αCH–Chx), 3.68–3.88 (m, 2H, δCH_2–Pro), 4.10–4.38 (m, 4H, CH_2Ph, αCH–Pro, CH), 6.58–6.68 (m, 2H, NH_2), 7.30–7.58 (m, 6H, Ph, NH), 7.82 (7.86, 8.25) (d, 1H, J = 7.91 Hz, NH). Anal. $C_{28}H_{39}N_5O_4S_2$ (C, H, N).

*6.1.3.1. (2S)-N-(2-Amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)-1-[(2R)-2-[(benzylsulfonyl)amino]-3-(3,4-dichlorophenyl)propanoyl]-2-pyrrolidinecarboxamide (**10**).* Using a general procedure described above, **10** was prepared from *N*-(benzylsulfonyl)-D-(3,4-dichlorophenyl)-alanyl-L-proline[21a] (140 mg, 0.29 mmol) and 4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrobromide (86 mg, 0.26 mmol); yield: 25 mg (15%), pale brown foam; IR (KBr): *v* 3326, 1654, 1522, 1448, 1317, 1152, 1128, 1031, 697, 544 cm^{-1}. MS (FAB): 636 (MH^+, 100%). Anal. $C_{28}H_{31}Cl_2N_5O_4S_2$ (C, H, N).

6.1.3.2. tert-*Butyl 2-[[(1R)-2-((2S)-2-{[(2-amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)amino]carbonyl}-pyrrolidinyl)-1-(cyclohexylmethyl)-2-oxoethyl](*tert-*butoxycarbonyl)-amino]acetate (**11**).* Using a general procedure described above, **11** was prepared from (2*S*) 1-((2R)-2-{(*tert*-butoxycarbonyl)[2-(*tert*-butoxy)-2-oxoethyl]amino}-3-cyclohexylpropanoyl)-proline [21b] (241 mg, 0.50 mmol) and 4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrobromide (149 mg, 0.45 mmol); yield: 185 mg (65%), white crystals; mp 99–101 °C. IR (KBr): *v* 3318, 2978, 2927, 2585, 1750, 1698, 1646, 1529, 1447, 1368, 1228, 1158 cm^{-1}. MS (FAB): 634 (MH^+, 100%). Anal. $C_{32}H_{51}N_5O_6S$ (C, H, N).

*6.1.3.3. (2S)-N-(2-Amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)-1-{(2R)-2-[(benzylsulfonyl)amino]-3-phenyl-propanoyl}-2-pyrrolidinecarboxamide (**12**).* Using a general procedure described above, **12** was prepared from *N*-(benzylsulfonyl)-D-phenylalanyl-L-proline (166 mg, 0.40 mmol) and 4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrobromide (120 mg, 0.36 mmol); yield: 100 mg (49%), pale yellow solid; mp 126–129 °C. IR (KBr): *v* 3362, 3192, 2926, 1646, 1522, 1454, 1315, 1152, 1124, 944, 751, 698, 547 cm^{-1}. MS (FAB): 568 (MH^+, 100%). Anal. $C_{28}H_{33}N_5O_4S_2$ (C, H, N).

*6.1.3.4. 2-{[(1R)-2-((2S)-2-{[(2-Amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)amino]carbonyl}-pyrrolidinyl)-1-(cyclohexylmethyl)-2-oxoethyl]amino}acetic acid dihydrochloride (**13**).* Dry HCl gas was bubbled into a solution of **11** (211 mg, 0.33 mmol) in glacial acetic acid (4 ml) for 1 h at room temperature. The acetic acid was removed in vacuo and the residue triturated several times with anhydrous ether. The white precipitate was filtered off and dried in vacuo over NaOH; yield: 167 mg (92%); mp 180–182 °C. IR (KBr): *v* 2929, 2288, 1742, 1639, 1548, 1448, 1227, 1116, 880, 710 cm^{-1}. MS (FAB): 478 [$(MH\text{-}2HCl)^+$, 100%]. Anal. $C_{23}H_{35}N_5O_4S \times 2HCl \times CH_3COOH$ (C, H, N).

6.1.3.5. tert-*Butyl (1R)-2-((2S)-2-{[(2-amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)amino]carbonyl}pyrrolidinyl)-1-benzhydryl-2-oxoethylcarbamate (**14**).* Using a general procedure described above, **14** was prepared from *N*-(*tert*-butyloxycarbonyl)-D-3,3-diphenylalanyl-L-proline [21a] (219 mg, 0.50 mmol) and 4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrobromide (149 mg, 0.45 mmol); yield: 191 mg (72%); mp 218–220 °C. Anal. $C_{32}H_{39}N_5O_4S \times 0.5H_2O$ (C, H, N).

*6.1.3.6. (2S)-1-[(2R)-2-Amino-3,3-diphenylpropanoyl]-N-(2-amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)-2-pyrrolidinecarboxamide dihydrochloride (**15**).* Dry HCl gas was bubbled into a solution of **14** (185 mg, 0.31 mmol) in glacial acetic acid (10 ml) for 1 h at room temperature, the acetic acid was removed in vacuo and the residue triturated several times with anhydrous ether. The white precipitate was filtered off and dried in vacuo over NaOH; yield: 169 mg (97%); mp 208–210 °C. IR (KBr): *v* 3390, 2974, 1641, 1449, 1347, 1242, 1112, 913, 753, 704 cm^{-1}. MS (FAB): 490 [(MH-2HCl)$^+$, 73%], 149 (100%). Anal. $C_{27}H_{31}N_5O_2S \times 2HCl \times CH_3COOH$ (C, H, N).

*6.1.3.7. (2R)-1-((2S)-2-{[(2-Amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)amino]-carbonyl}pyrrolidinyl)-3-(3,4-dichlorophenyl)-1-oxo-2-propanamine dihydrochloride (**16**).* Using a general procedure described above, *tert*-butyl (1*R*)-2-((2*S*)-2-{[(2-amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)amino]carbonyl}pyrrolidinyl)-1-(3,4-dichlorobenzyl)-2-oxoethylcarbamate (**22**) was prepared from *N*-(*tert*-butoxycarbonyl)-D-(3,4-dichlorophenyl)-alanyl-L-proline [21a] (474 mg, 1.1 mmol) and 4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrobromide (331 mg, 1.00 mmol); yield: 448 mg (77%), pale yellow amorphous solid; mp 116–119 °C. IR (KBr): *v* 3409, 2977, 1637, 1529, 1444, 1367, 1250, 1165, 1029, 753, 679 cm^{-1}. MS (FAB): 582 (MH^+, 49%), 70 (100%). Anal. $C_{26}H_{33}Cl_2N_5O_4S$ (C, H, N).

The crude **22** (400 mg, 0.69 mmol) was dissolved in glacial acetic acid (10 ml), then dry HCl gas was introduced into the solution for 1 h at room temperature. The mixture was evaporated in vacuo and the residue triturated with anhydrous ether to give **16** as pale yellow solid; yield: 366 mg (96%) mp 166–169 °C. IR (KBr): *v* 2973, 1643, 1469, 1358, 1261, 1132, 1032, 878, 826, 713, 586 cm^{-1}. MS (FAB): 483 [(MH-2HCl)$^+$, 100%]. Anal. $C_{21}H_{25}Cl_2N_5O_2S \times 2HCl$ (C, H, N).

*6.1.3.8. (2R)-N-{2-[(2-Amino-4,5,6,7-tetrahydro-1,3-benzo-thiazol-6-yl)amino]-2-oxoethyl}-2-[(benzylsulfonyl)-amino]-3-phenylpropanamide (**17**).* Using a general procedure described for the synthesis of **9** above, **17** was prepared from *N*-(benzylsulfonyl)-D-phenylalanyl-glycine (150 mg, 0.40 mmol) and 4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrobromide (119 mg, 0.36 mmol); yield: 70 mg (37%), white solid; mp 119-122 °C. IR (KBr): *v* 3346, 2926, 1655, 1522, 1455, 1371, 1314, 1233, 1126, 956, 750, 698, 543, 487 cm^{-1}. MS (FAB): 528 (MH^+, 88%), 91(100%). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.55–1.87 (m, 2H, CH_2), 2.20–2.60 (m, 4H, 2 × CH_2,), 2.80 (dd, BX part of ABX system, 1H, J_{BX} = 9.42 Hz, J_{AB} = 13.66 Hz, CH_2–Phe), 3.02 (dd, AX part of ABX system, 1H, J_{AX} = 5.09 Hz, J_{AB} = 13.76 Hz, CH_2–Phe), 3.82–4.20 (m, 5H, CH_2–Gly, $PhCH_2SO_2$, CH), 4.15–4.30 (m, 1H, CH), 6.65 (s, 2H, NH_2), 7.15–7.40 (m, 10H, 2 × Ph), 7.67 (d, 1H, *J* = 9.04 Hz, $NHSO_2$), 7.95–8.00 (m, 1H, NH), 8.54 (t, 1H, *J* = 5.66 Hz, NH–Gly) ppm. Anal. $C_{25}H_{29}N_5O_4S_2 \times H_2O$ (C, H, N).

*6.1.3.9. (2R)-N-{(1S)-2-[(2-Amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl)amino]-1-methyl-2-oxoethyl}-2-[(benzyl-sulfonyl)amino]-3-phenylpropanamide (**18**).* Using a general procedure described above, **18** was prepared from *N*-(benzylsulfonyl)-D-phenylalanyl-alanine (195 mg, 0.50 mmol) and 4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrobromide (149 mg, 0.45 mmol); yield: 90 mg (37%), white solid; mp 122–125 °C. IR (KBr): *v* 3314, 3200, 2929, 1654, 1523, 1455, 1371, 1315, 1231, 1152, 1126, 952, 750, 698, 545 cm^{-1}. MS (FAB): 542 (MH^+, 100%). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.03–1.15 (m, 3H, CH_3–Ala), 1.57–1.89 (m, 2H, CH_2), 2.20–2.50 (m, 3H, CH_2, CH), 2.57–2.98 (m, 3H, CH_2Ph, CH), 3.80–4.33 (3 × m, 5H, $PhCH_2SO_2$ + 3 × CH), 6.62 (s, 2H, NH_2), 7.11–7.38 (m, 10H, 2×Ph), 7.58–7.66 (m, 1H, $NHSO_2$), 7.84–7.98 (m, 1H, NH), 8.26–8.38 (m, 1H, NH–Ala) ppm. Anal. $C_{26}H_{31}N_5O_4S_2 \times H_2O$ (C, H, N).

*6.1.3.10. (2S)-N-(2-Amino-1,3-benzothiazol-6-yl)-1-{(2R)-2-[(benzylsulfonyl)amino]-3-phenylpropanoyl}-2-pyrrolidi-necarboxamide (**19**).* Compound **19** was prepared by coupling *N*-(benzylsulfonyl)-D-phenylalanine (180 mg, 0.56 mmol) and *N*-(2-amino-1,3-benzothiazol-6-yl)-pyrrolidinecarboxamide dihydrochloride (**23**) (169 mg, 0.50 mmol) using a procedure described for the synthesis of **9**; yield: 100 mg (35%), pale yellow solid; mp 130–133 °C. IR (KBr): *v* 3314, 1637, 1526, 1466, 1407, 1307, 1227, 1150, 1123, 934, 819, 748, 698, 544, 488 cm^{-1}. MS (FAB): 564 (MH^+, 100%). Anal. $C_{28}H_{29}N_5O_4S_2 \times H_2O$ (C, H, N).

*6.1.3.11. (2R)-N-{2-[(2-Amino-1,3-benzothiazol-6-yl)amino]-2-oxoethyl}-2-[(benzylsulfonyl)amino]-3-phenylpropa-namide (**20**).* Using a procedure described for the synthesis of **9**, compound **20** was prepared from *N*-(benzylsulfonyl)-D-phenylalanine (400 mg, 1.25 mmol) and 2-amino-*N*-(2-amino-1,3-benzothiazol-6-yl)-acetamide dihydrochloride (**24**) (336 mg, 1.14 mmol); yield: 170 mg (29%), yellow solid; mp 127–130 °C. IR (KBr): *v* 3351, 1639, 1530, 1466, 1408, 1307, 1123, 958, 818, 697, 539 cm^{-1}. MS (FAB): 524 (MH^+, 100%). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 2.80 (dd, BX part of ABX system, 1H, J_{BX} = 9.42 Hz, J_{AB} = 13.66 Hz, CH_2–Phe), 3.02 (dd, AX part of ABX system, 1H, J_{AX} = 5.09 Hz, J_{AB} = 13.66 Hz, CH_2–Phe), 3.81–4.11 (m, 4H, 2 × CH_2), 4.22 (m, 1H, CH), 7.15–7.40 (m, 13H, 2 × Ph, 3 × CH), 7.67 (d, 1H, *J* = 9.04 Hz, $NHSO_2$), 7.95–8.00 (m, 1H, NH), 8.54 (t, 1H, *J* = 5.66 Hz, NH–Gly), 9.85 (s, 2H, NH_2) ppm. Anal. $C_{25}H_{25}N_5O_4S_2 \times H_2O$ (C, H, N).

*6.1.3.12. (2S)-N-[(2-Amino-4,5,6,7-tetrahydro-1,3-benzo-thiazol-6-yl)methyl]-1-{(2R)-2-[(benzylsulfonyl)amino]-3-cyclohexylpropanoyl}-2-pyrrolidinecarboxamide (**21**).* Using a general procedure described above for the synthesis of **9**, the compound **21** was prepared from *N*-(benzylsulfonyl)-D-(β-cyclohexyl)-alanyl-L-proline (186 mg, 0.44 mmol) and 6-aminomethyl-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrobromide (138 mg, 0.40 mmol); yield:

183 mg (78%); white foam. IR (KBr): *v* 3356, 2924, 1648, 1523, 1444, 1311, 1141, 782, 699, 546 cm^{-1}. MS (FAB): *m/z* (%) 588 (MH^+, 100). Anal. $C_{29}H_{41}N_5O_4S_2$ (C, H, N).

*6.1.3.13. 2-Amino-*N*-(2-amino-1,3-benzothiazol-6-yl)-acetamide dihydrochloride (**24**).* A stirred solution of *N*-(*tert*-butoxycarbonyl)glycine (1.31 g, 7.5 mmol) in *N,N*-dimethylformamide (6 ml) was cooled to –15 °C and treated successively with ethyl chloroformate (0.79 ml, 8.25 mmol) and triethylamine (1.15 ml, 8.25 mmol). After 1 h at –15 °C benzothiazol-2,6-diamine (1.24 g, 7.5 mmol) was added and the mixture was kept at 0–5 °C overnight. The precipitate was filtered off and the filtrate was evaporated under reduced pressure. The residual oil was triturated with ether to give crude *tert*-butyl 2-[(2-amino-1,3-benzothiazol-6-yl)amino]-2-oxoethylcarbamate (**25**) as a pale yellow solid foam; yield: 0.51 g (21%). IR (KBr): *v* 3303, 2974, 1672, 1531, 1472, 1411, 1230, 1159, 1124, 816 cm^{-1}. MS (FAB): 323 (MH^+, 100%). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.40 (s, 9H, *tert*-Bu), 3.70 (d, 2H, *J* = 6.03 Hz, CH_2–Gly), 7.00 (t, 1H, *J* = 6.03 Hz, NH–Gly), 7.20–7.40 (m, 3H, 3 × CH–Ar), 7.99 (s, 1H, NH), 9.83 (s, 2H, NH_2) ppm.

The crude **25** (487 mg, 1.51 mmol) was dissolved in glacial acetic acid (20 ml) and then dry HCl gas was introduced into the solution for 1 h at room temperature. The mixture was evaporated in vacuo and the residue triturated with anhydrous ether to give **24** as pale yellow solid; yield: 360 mg (81%) mp 189–192 °C. IR (KBr): *v* 3240, 3050. 1697, 1647, 1596, 1560, 1491, 1439, 1318, 1237, 946, 836, 670 cm^{-1}. MS (FAB): 223 [$(MH\text{-}2HCl)^+$, 100%]. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 3.81 (s, 2H, CH_2–Gly), 7.50 (m, 1H, CH–Ar), 7.60 (m, 1H, CH–Ar), 7.95 (s, 1H, NH), 8.22 (m, 1H, CH–Ar), 8.32 (s br, 3H, NH_3^+), 9.60 (s br, 2H, NH_2) ppm. Anal. $C_9H_{10}N_4OS \times 2HCl$ (C, H, N).

Using the same procedure, (2*S*)-*N*-(2-amino-1,3-benzothiazol-6-yl)-pyrrolidinecarboxamide dihydrochloride (**23**) was prepared from *N*-(*tert*-butoxycarbonyl)-L-proline and benzothiazol-2,6-diamine.

6.1.4. X-ray crystallography

6.1.4.1. Crystallization and data collection. Human α-thrombin was obtained from Roche (Mannheim, Germany) as a lyophilisate. Sulfated hirugen (54–65) with the sequence H-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr (SO_3H)-Leu-Gln-OH was obtained from Bachem (Heidelberg, Germany) as a lyophilisate. Thrombin crystals were prepared as described previously [22]. The crystallization was carried out using the hanging drop vapor diffusion technique. Macroseeding was necessary to obtain crystals of a size adequate for the diffraction experiment. Crystals were soaked in a solution containing 1 mM of inhibitor overnight and then mounted on a loop for data collection. A single crystal was shock frozen at 100 K and a complete data set was taken using the MAR image plate system.

6.1.4.2. Structure solution and refinement. Diffraction data were indexed and integrated using the MOSFLM v. 6.11 program [23] and scaled with the SCALA software [24]. The exact orientation and positions were determined with the Patterson search techniques using the AMORE program [25] and α-thrombin–hirugen coordinates (1HGT.pdb) as a replacement model. Initial models of the inhibitors were constructed and minimized using the SYBYL 6.7. program package. Subsequently, the inhibitors were fitted into the electron density map manually and the crystallographic refinement was performed using the CNS version 1.0. [26].

6.1.5. Biological assays

6.1.5.1. Amidolytic enzyme assay. Spectrophotometric enzyme tests were performed in microtiter plates in a final volume of 125 µl. Thrombin was tested at 1 nM with the substrate S-2366 at 100 nM final concentration and factor Xa at 3 nM with the substrate S-2222 at 200 nM final concentration. Trypsin was tested at 6 nM using the substrate S-2251 at 200 nM. Inhibitors were dissolved in DMSO (concentration of stock solutions: 10 mmol/l) and tested at 100 µM as the highest concentration. Inhibitors were diluted using HNPT buffer pH 7.8 consisting of HEPES 100 mM, NaCl 140 mM, PEG 6000 0.1% and Tween 80 0.02%. The reaction kinetics were linear both with the time and the concentration of the enzyme. Full concentration–response curves were run for inhibitors showing >50% inhibition for any enzyme at 100 µM. Apparent K_i values were calculated according to Cheng and Prusoff based on IC_{50} [17a], or from a relation between reaction velocity in the absence and presence of inhibitor using the relevant K_m [17b]. The K_m for the substrates used were determined under the test conditions with at least five substrate concentrations ranging from 0.5 to 15 times K_m [27] according to Eadie [28]. The K_m was 108 µM for the substrate S-2366, 613 µM for the substrate S-2222 and 430 µM for the substrate S-2251.

6.1.5.2. Coagulation assays. The anticoagulant effect of thrombin inhibitors was measured by TT, APTT and PT assays. Inhibitors were added to normal pooled plasma over a range of concentrations and clotting was recorded in an automated coagulometer (Fibrintimer, Dade/Behring). The concentration of inhibitor that doubled the clotting time was determined for each assay. *Normal pooled plasma*: Venous blood from 11 apparently healthy volunteers was collected (one part 0.11 mol/l sodium citrate solution with nine parts of blood) and centrifuged immediately at 2000 × *g* for 30 min at 4 °C. Plasma was removed, pooled and stored in aliquots at –70 °C until use. For *TT determination*, inhibitors (10 µl working solution, concentrations from 2.5 to 100 µM) and pooled plasma (90 µl) were incubated at 37 °C for 5 min whereupon thrombin (200 µl) was added. Clot formation was measured in a coagulometer in duplicate. For *APPT determination*, inhibitors (10 µl working solution, concentrations from 5 to 100 µM) and pooled plasma (90 µl) were incubated

at 37 °C for 5 min whereupon Pathromtin (Dade/Behring) (100 µl) was added and the sample was incubated for another 2 min at 37 °C. The addition of calcium chloride (100 µl) triggered the coagulation process and clot formation was detected with a coagulometer in duplicate. For *PT determination*, inhibitors (10 µl working solution, concentrations from 5 to 100 µM) and pooled plasma (90 µl) were incubated at 37 °C for 5 min whereupon thromboplastin (Thromborel S, Dade/Behring) (200 µl) was added. Clot formation was measured in a coagulometer in duplicate.

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